FUNCTIONAL PROFILING OF MICRORNAS IN STALLIONS

An Undergraduate Research Scholars Thesis

by

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ABSTRACT

Functional profiling of microRNAs in stallions. (May 2013)

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MicroRNAs (miRNAs) are short non-coding RNAs that act as post-transcriptional regulators of gene expression in eukaryotic genomes and are thought to be critically involved in many biological processes. While the functions of sperm miRNAs in equine biology are yet to be determined, studies in mouse and humans suggest that sperm miRNAs regulate gene expression in the zygote and can indicate the status of male fertility. The aim of this study was to characterize the expression profiles of selected sperm miRNA in equine tissues and compare their expression levels in the sperm and testes of fertile/sexually mature and subfertile/sexually immature stallions. From sperm RNA-seq data, we selected 6 highly expressed miRNAs: miR-34b, -34c, -191, -223, -1248 and -1905c. Total RNA enriched with miRNAs was extracted from 10 adult tissues, sperm of 3 fertile and 3 subfertile stallions, and testes of five 1-year old and five 3-year old stallions. The RNA was polyadenylated, reverse transcribed into srcDNA, and examined through RT-PCR and qRT-PCR. Reverse transcriptase PCR on a panel of adult male tissues revealed ubiquitous expression of the 6 miRNAs, whereas transcription of miR-34c, -223, and -1905c was elevated in testes and sperm. Additionally, we showed that stallion sperm and testes contain transcripts of mature sperm-enriched tRNA-derived small RNAs (mse-tsRNAs), which is a novel finding for the horse. A pilot study was conducted to quantify the expression of

miR-34c and miR-1905c in the sperm of fertile and subfertile stallions. While the expression levels varied between individuals and the two fertility phenotypes, a significantly (p=0.04) elevated expression of miR-34c was observed in the subfertile group. Finally, due to the overall high expression of miR-1905c in sperm, its expression was qualified and quantified in the testes of 1-year old and 3-year old stallions. miR-1905c was expressed in all testes samples and no significant differences in expression level were observed between immature and maturing testes. Because the number of stallions was limited, the current results remain preliminary and further experimentation will be required. Nevertheless, the discovery of miRNAs in stallion sperm might lead to a new direction in the search of biomarkers for stallion fertility.

DEDICATION

To my parents, Dr. Yu Feng Wang and Mrs. Huey Ying Wang, who have supported me all the way and have helped me get to where I am today. Also, to my sister, Amy Michelle Wang, and my brother, Austin Christiansen Wang, thank you for all the years of having my back.

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I would like to express my deepest gratitude to my advisor, Dr. Terje Raudsepp, for all of her guidance in this project and in the lab. Also, to graduate students Ms. Sharmila Ghosh, Mr. Felipe Avila, and Ms. Alex Trott, I am grateful that you all put up with me on a routine basis. And finally, an enormous thank you goes to Ms. Joana Rocha for teaching me the fine art of gel loading. I would not be here at this point without the support of these individuals.

NOMENCLATURE

bp	base pair
miRNA	microRNA
mRNA	messenger RNA
mse-tsRNA	mature-sperm-enriched tRNA-derived small RNA
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time PCR
RISC	RNA-induced silencing complex
RT	reverse transcription
RTase	reverse transcriptase
RT-PCR	reverse transcription PCR
srcDNA	short RNA complementary DNA
Tris/Borate/EDTA	TBE
Tm	melting temperature
tRNA	transfer RNA

CHAPTER I

INTRODUCTION

MicroRNAs (miRNAs) are among the many types of non-coding RNAs found in eukaryotic cells and as the name suggests, are short RNA strands of around 20 to 24 nucleotides in length that do not code for amino acids (Prichard et al. 2012). Since the discovery of various small non-coding RNAs such as small interfering RNAs, piwi-interacting RNAs, and microRNAs, the purpose of these particular RNAs in eukaryotic genomes was mostly unclear and many of these RNAs were thought of as unusable "junk" RNA (Prichard et al. 2012). However, these various small RNAs have now been recognized as essential regulators in eukaryotic cell development and maintenance. MicroRNAs are distinguished from other small non-coding RNAs through their biogenesis, size, and regulatory functions.

MicroRNA Biogenesis and Structure

The genes coding for miRNAs are situated in introns of genes, in exons of non-coding genes, and in intergenic regions (Schmittgen et al. 2007). Mature miRNA strands are processed in a multi-step fashion from a hairpin-structured 200-300 nucleotide long primary miRNA (pri-miRNA), which is first cleaved in the nucleus by the enzyme Drosha in a Microprocessor complex to form a 70 to 100 nucleotide long, hairpin-structured, precursor miRNA (pre-miRNA) (Papaioannou et al. 2010). The pre-miRNA is then exported from the nucleus by Exportin-5 into the cytoplasm (Papaioannou et al. 2010). Next, an enzyme called Dicer cuts the double-stranded hairpin-structured pre-miRNA into a miRNA duplex where it forms with the Argonaute protein a preliminary RNA-induced silencing complex (RISC) (Papaioannou et al. 2010). An RNA

helicase then proceeds to unwind the duplex and one strand is degraded while the other becomes the mature, 20 to 24 nucleotide long, single-stranded miRNA that remains in the mature RISC complex (Papaioannou et al. 2010). Biogenesis and function of miRNA is summarized below (Figure 1).

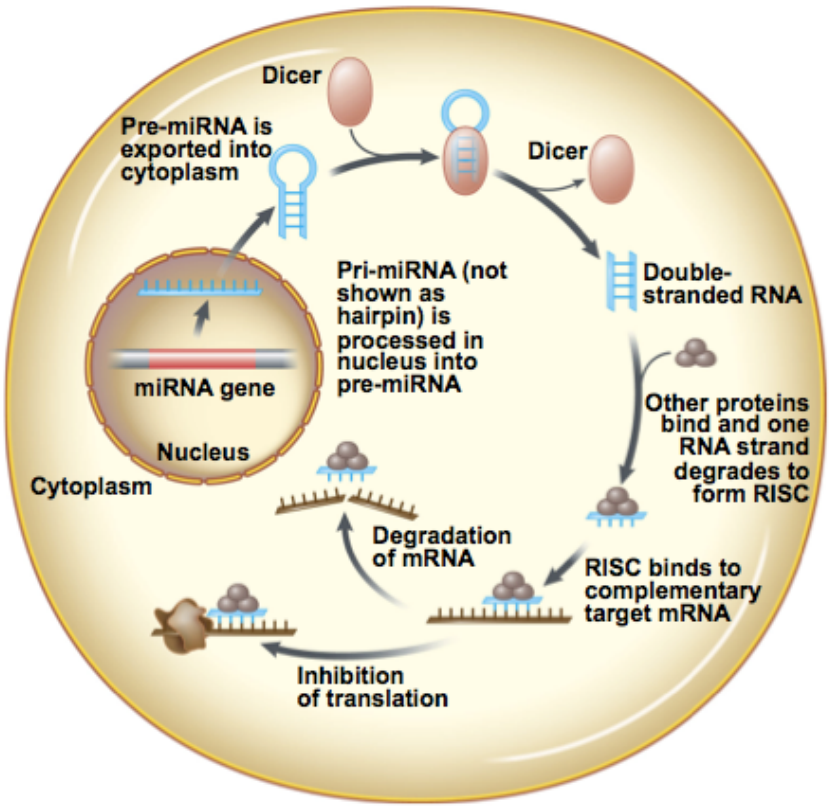

Figure 1. MicroRNA biogenesis and functions. A diagram summarizing the process of miRNA biogenesis and functions (adapted from Cummings 2010).

MicroRNA Functions

The miRNA is essentially utilized as a template for the RISC to seek out a complementary messenger RNA (mRNA) sequence to bind to. Studies have demonstrated that miRNAs can regulate post-transcriptional gene expression negatively by binding to mRNAs in their RISC,

where the target mRNA is ultimately cleaved, degraded, or inhibited, or positively through targeting of gene promoters in a similar manner (McCallie et al. 2010). Through these actions, particular target genes are deactivated, activated, or expressed differently, and miRNAs are responsible for the regulation of thousands of genes in eukaryotic organisms (Pritchard et al. 2012). Current studies show that miRNAs exist in nearly all mammalian cell types, each differentiated for the specific cell type and each regulating hundreds to thousands of protein-coding genes in the mammalian genome (McCallie et al. 2010). The genes that miRNAs regulate are pertinent in cell development and communication processes such as metabolism, stem cell differentiation, apoptosis, gene methylation, and a number of other important cellular functions (Chen et al. 2005).

Functions of miRNAs in Reproduction and Development

Among the multitude of miRNAs in the eukaryotic genome, there are those regulating post-transcriptional control after meiosis in spermatogenesis, the process of creating new, viable sperm in males (Papaioannou et al. 2010). When small non-coding RNAs were discovered in sperm, it was conjectured that small RNAs, such as miRNAs, might function as important gene regulators during sperm chromatin packaging, delivery, and early embryonic development (Krawetz et al. 2011, Kawano et al. 2012). Thus, many of the issues with spermatogenesis-linked male infertility have been speculated to arise from problems with gene regulation such as miRNA regulation of nuclear condensation in sperm (Papaioannou et al. 2010). It was found that the loss of miRNAs in the supporting or germ cells of the testis had detrimental effects on male fertility in mice (Papaioannou et al. 2010). There is also strong evidence that certain miRNAs derived from sperm are responsible for early development as found in mice (Liu et al. 2011) and

humans (Krawetz et al. 2011, Sendler et al. 2013). A miRNA, designated miR-34c, was detected in murine sperm and zygotes, but not in oocytes, and was found to be responsible for regulating genes required for the first cleavage division (Liu et al. 2011). Inhibition of miR-34c resulted in halted development in single-cell mouse zygotes (Liu et al. 2011). miR-34c is also among the most abundant transcripts in human sperm and is thought to regulate the expression of *DLL1* and *NOTCH1* - genes known to play an important role in segmentation and somite formation in vertebrates (Krawetz et al. 2011). Another abundant miRNA in human sperm, miR-181c, modulates the expression levels of *CARM1*, a key regulator of critical pluripotency factors in human and mouse embryonic stem cells and blastomeres (Sendler et al. 2013). The importance of miRNAs in early development is further exemplified by experiments where targeted elimination of Dicer utilized in miRNA biogenesis resulted in incomplete embryo development (Rosenbluth et al. 2013). Levels of miRNA-targeted mRNAs were also seen to be higher in mouse embryos than those found in mature mice, which supports the importance of miRNA regulation in embryo development (McCallie et al. 2010). Thus, the fate of developing embryos and overall male fertility lies largely in the regulatory actions of miRNAs. It can be seen that disruption of miRNA production and expression has detrimental effects in overall male fertility and embryonic development. Research regarding miRNA and its link to fertility has been performed on few species such as humans and mice but data on several other animals such as horses has not yet been collected or examined largely to date.

MicroRNAs in horses

The studies of miRNAs in horses are limited. The first equine miRNAs, miR-433 and miR-127, were isolated in 2009 (Song and Wang, 2009), followed by an *in silico* analysis of the horse

genome sequence and genome-wide discovery of 407 equine miRNAs (Zhou *et al.*, 2009). Very recently over 80 miRNAs were found by RNA-seq in stallion sperm (Das et al. 2013) suggesting that like in humans and mice, miRNAs might regulate sperm functions, fertilization and early embryonic development also in horses.

Project Goals and Objectives

This project was initiated with an aim to examine the possible effects that miRNA expression may have on stallion fertility. Several miRNA genes were recently found to have relatively high expression levels in stallion sperm and were selected for examination as potential gene regulators associated with stallion fertility (Das et. al 2013). Small non-coding RNAs such as miRNA exhibit roles that are pertinent to various critical functions in eukaryotes. Many factors affect essential genetic aspects of reproduction such as gene silencing and alteration and miRNAs have been observed to contribute to such crucial processes in eukaryotic genomes. We hypothesize that expression levels of sperm-derived miRNA are indicative of a stallion's fertility and thus, stallions known to be infertile or sexually immature should exhibit deviant expression levels of miRNAs. The immediate goal of this project was to determine expression profiles of selected miRNAs in equine tissues and in the sperm of fertile/mature and subfertile/immature stallions. Our long-term goal is to identify miRNAs that can serve as biomarkers for evaluating stallion fertility. The goal of this research was accomplished through the following specific objectives:

Objective #1: Determine expression profiles of six selected sperm-derived miRNAs by qualitative reverse transcriptase PCR (RT-PCR) in a panel of adult male horse tissues.

Objective #2: Quantify expression levels of two sperm-derived and abundant miRNAs, **miR-34c**

and **miR-1905c,** by qRT-PCR in the sperm and testes of fertile/reproductively mature stallions and subfertile/reproductively immature stallions.

Objective #3: Determine the presence of mature-sperm-enriched transfer RNA-derived small RNAs (mse-tsRNAs), a novel class of small regulatory RNAs, in stallion sperm and testes. Notably, mse-tsRNAs were recently discovered to be highly abundant in mouse sperm (Peng et al. 2012).

CHAPTER II

METHODS

Until recently, identification and quantification of miRNAs was hindered due to the relatively small size and low quantities of miRNAs in the mammalian genome. However, recent technological advancements which are also utilized in this project have emerged to remedy these issues. The overall process is illustrated in Figure 2. In general, after extraction of miRNAs containing total RNA, the mature miRNA strands are extended through the use of poly-A polymerase (Fig. 2.2), reverse transcribed into srcDNA with a reverse transcription (RT) primer and reverse transcriptase (RTase; Fig. 2.3), and then analyzed for expression levels through quantitative real-time PCR (qRT-PCR) (Lu et al. 2005). Typically, miRNAs sequences are amplified using a miRNA-specific forward primer and a universal reverse primer (Fig. 2.4).

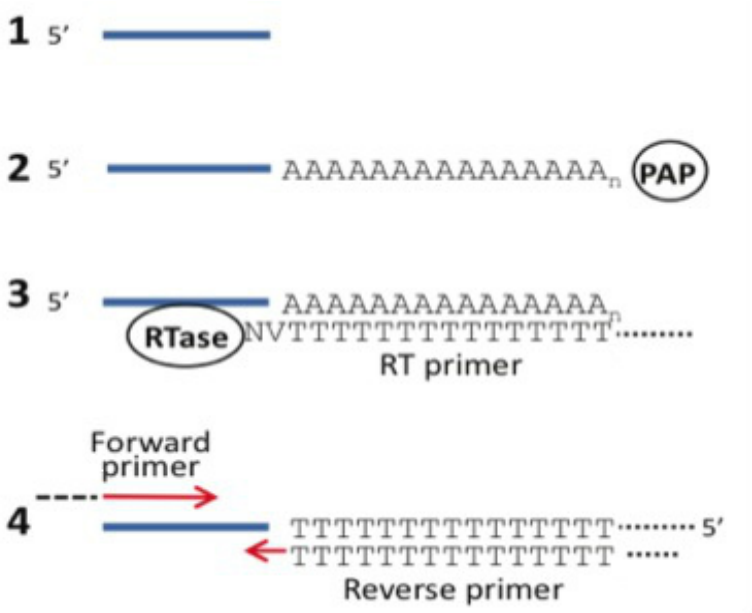

Figure 2. Flow scheme of miRNA-specific PCR (Ballcells et al. 2011)

Thus, profiling of miRNAs is now a staple method for the discovery and quantitation of miRNAs responsible for cell processes and even certain diseases at the genetic level (Schmittgen et al. 2008).

Bioinformatics analysis of miRNA sequences and primer design

MicroRNAs for this study were selected from recent RNA-sequence data for stallion sperm transcriptome (Das et al. 2013) and comprised miRNAs that are known to have high expression levels in stallion sperm (Table 1).

Table 1. Six most abundant microRNAs in stallion sperm (Das et al. 2013)

miRNA symbol	Chromosomal location; chr:bp	Expression level (highest average coverage (AC) value)	Accession No (NCBI)	Accession No (miRBase)
miR-1248	19:24793832-24794009	536.83	NR_032967	MI0012839
miR-34b	7:20101184-20101217	336.58	NR_032870	MI0012741
miR-34c	7:20101727-20101755	336.0	NR_032871	MI0012742
miR-223	X:48489279-48489317	295.64	NR_033081	MI0012953
miR-191	16:38001162-38001193	160.38	NR_032953	MI0012825
miR-1905c	7:541073-541099	124.94	NR_032865	MI0012736

The miRNA sequences were obtained from MirBase and NCBI databases and PCR primers were designed using the Exiqon miRNA PCR primer designer (http://www.exiqon.com/miRNA-qpcr-designer). Primer sequences are shown in Table 2.

Table 2. MicroRNA primer sequences

miRNA	Forward primer 5'-3'
miR-1248	GCGGCGGTCCTTCTTGTATAAG
miR-34b	GCGGCGGAGGCAGTGTAATTAG
miR-34c	GCGGCGGAGGCAGTGTAGTTAG
miR-223	GCGGCGGTGTCAGTTTGTCAAA
miR-191	GCGGCGGCAACGGAATCCCAAAAG
miR-1905C	GCGGCGGCACCACCAGCCCCAC
Universal Reverse primer	ATCCAGTGCAGGGTCCGAGG

Total RNA extraction from stallion tissues and sperm

Small RNA-containing total RNA was isolated from the following types of samples:

a) 10 adult horse tissues: brain, heart, kidney, liver, lung, spleen, skeletal muscle, ovary, testis, sperm.

b) Sperm of three fertile and three subfertile stallions.

c) Testes of five 1-year old (sexually immature) and five 3-year old (sexually mature) stallions.

The RNA was extracted using the mirVana miRNA isolation kit (Ambion) and the manufacturer's protocol. For the sperm miRNA isolation, the ejaculates were first purified by discontinuous gradient centrifugation through a 40% silanized silica particle solution EquiPure™ Top Layer (Nidacon International, Mölndal, Sweden) as described by Das et al. (2010). The purified sperm were collected in a microfuge tube on ice and mixed with 450 µL of Lysis/Binding Buffer and 45 µL of miRNA Homogenate Additive, and vortexed for 30 sec. The mixture was homogenized with a 27.5 gauge needle syringe to lyse the cells and the mixture was left on ice for 10 min. Next, 450 µL of Acid Phenol: $ChCl_3$ (5:1 Solution) was added into the tube, vortexed for 30 sec, and centrifuged for 15 min at 11,000 rcf. The aqueous phase was

transferred into a new tube and mixed with 565 μL of 100% ethanol. The filter cartridge and collection tube were assembled and the mixture was moved into the filter cartridge. Next, the tube was centrifuged for 15 sec at 10,000 rcf to pass the fluid through the filter cartridge with the supernatants discarded. Thereafter, 700 μL of miRNA Wash Solution 1 was added into the filter cartridge and the tube was centrifuged for 10 sec at 10,000 rcf with the supernatants discarded. The step was repeated twice with 500 μL of miRNA Wash Solution 2/3. The RNA sample was eluted with 50 μL of nuclease-free water and the collection tube was centrifuged for 1 min at 16,100 rcf. The final RNA solution was transferred into a fresh microfuge tube.

Total RNA polyadenylation

The small RNA-containing total RNA from the tissues was polyadenylated and reverse transcribed into small RNA cDNAs (srcDNAs) using adapter attached oligo-dT primers. A polyadenylation mix for the appropriate number of reactions needed was prepared. Each reaction contained 16 μL of polyadenylation mix consisting of the following reagents: 10 μL of 5x EAP Buffer, 5 μL of 2.5 mM $MnCl_2$, 0.5 μL of 10 mM cATP, and 0.5 μL of Poly-A Polymerase; and finally 34 μL of RNA to make a total reaction volume of 50 μL. The reaction tubes were then placed into the thermocycler for 1 hr at 37 ^{o}C to anneal Poly-A tails to the RNA strands.

Reverse transcription of poly-A RNA into srcDNA

The poly-A RNA samples were converted into srcDNA through reverse transcription (RT). The first reverse transcription mix was prepared to prime the Poly-A RNA strand for reverse transcription. Each reaction contained 4 μL of the first reverse transcription mix consisting of the

following reagents: 3 µL of 25 mM RT primer and 1 µL of 10 mM dNTPs; and finally 6 µL of Poly-A RNA to make a total reaction volume of 10 µL. The reaction tubes were centrifuged at 13,000 rcf for 10 sec and incubated in a thermocycler for 5 min at 65 °C to anneal RT primers onto the Poly-A RNA strands. Next, a second reverse transcription mix was prepared to elongate the srcDNA strand. Each reaction contained 11 µL of the second reverse transcription mix consisting of the following reagents: 4 µL of nuclease-free water, 4 µL of 5x FS Buffer, 1 µL of 0.1 M DTT, 1 µL of RNase Out, and 1 µL of SuperScript® III Platinum® Taq DNA Polymerase (Invitrogen); and finally 10 µL of the first reverse transcription mix to make a total reaction volume of 21 µL. The reaction tubes were centrifuged at 13,000 rcf for 10 sec and incubated in a thermocycler for 50 min at 50 °C, and for 5 min at 85 °C. The samples were then chilled on ice to inactivate the reverse transcriptase. Finally, 1.5 U of RNase H (Promega) was added to remove the small RNAs. The samples were purified using the QIAquick® Spin PCR purification kit (Qiagen) in a final elution volume of 100 µL. The srcDNA concentration was measured using a NanoDrop2000 (Thermo Scientific) spectrophotometer. All srcDNA samples were diluted to the same concentration of 50 ng/µL.

Qualitative RT-PCR of 6 selected miRNAs in a panel of horse tissues

The presence or absence of a particular miRNA in different equine tissues was observed through RT-PCR and gel electrophoresis. Each PCR reaction contained 8 µL of PCR mix consisting of the following reagents: 6.05 µL of nuclease-free water, 1 µL of 10x PCR Buffer with 15 mM $MgCl_2$, 0.3 µL of a miRNA specific forward primer, 0.3 µL of a universal reverse primer, 0.1 µL of 20 mM dNTPs, and 0.25 µL of JumpStart REDTaq Polymerase (Sigma Aldrich); and 2 µL of template srcDNA to make a total reaction volume of 10 µL. The RT-PCR reactions were carried

out in a thermocycler with the annealing temperature set at 54 °C. A horse tissue panel, including srcDNA from brain, heart, kidney, liver, lung, spleen, skeletal muscle, ovary, testes, and sperm were examined with each miRNA primer shown in Table 1. The samples were analyzed using gel electrophoresis on a 3% agarose gel. The presence of amplification verified the presence of a miRNA in a particular equine tissue.

Quantitative real-time PCR (qRT-PCR) of miR-34c and miR-1905c in stallion sperm

Two miRNAs, miR-1905c and miR-34c, found to be sperm-predominant by qualitative PCR analysis were further analyzed by qRT-PCR. Quantitative RT-PCR was performed using SYBR Green PCR Master Mix (Roche), 1 μL of srcDNAs, and 10 μM of the forward and reverse primers in a 20 μL reaction volume. The sperm srcDNA from 3 fertile and 3 subfertile stallions were examined with primers corresponding to miR-1905c and miR-34c. The reactions were carried out in LightCycler 480 (Roche Diagnostics) at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, primer annealing at 54 °C for 30 sec, and strand elongation at 60 °C for 30 sec. A housekeeping gene *GAPDH* was utilized as the reference. The results were analyzed with LightCycler 480 Software v1.5 by calculating $\log_2^{-\Delta\Delta Ct}$. The *p*-value was calculated by performing student's t-test and $p < 0.05$ was considered significant.

Qualitative and quantitative RT-PCR of miR-1905c in stallion testes

The expression of miR-1905c, which was found to be in higher amounts than miR-34c in sperm, was analyzed by qualitative reverse transcriptase PCR (RT-PCR) and quantitative real-time PCR

(qRT-PCR) in the testes of five 1-year old and five 3-year old stallions. The former were treated as “reproductively immature/subfertile” and the latter as “reproductively mature/fertile”. The procedures were identical to the RT-PCR and qRT-PCR protocols as described above.

Qualitative RT-PCR of mse-tsRNAs in stallion sperm and testes

The presence or absence of mse-tsRNAs Family 1 and Family 2 (Peng et al. 2012) was examined in stallion sperm and testes srcDNA through RT-PCR. The procedure was identical to the above described RT-PCR protocol, only that the primers for mse-tsRNA Family 1 and Family 2 were used and the samples only included one sperm and testis srcDNA.

CHAPTER III

RESULTS

<u>Objective #1:</u> Determine expression profiles of six selected sperm-derived miRNAs by qualitative reverse transcriptase PCR (RT-PCR) in a panel of adult male horse tissues.

Reverse transcriptase PCR with primers for miR-34b, -34c, -191, -223, -1248, and -1905c showed that all six miRNAs are ubiquitously expressed in the 10 adult equine tissues examined (Fig. 3). Some miRNAs gave two distinct PCR products indicating the presence of both the mature miRNA (lower band) and pre-miRNA (upper band) in these tissues. For example, miR-34c had two PCR products in spleen and sperm, miR-191 in kidney and spleen, miR223 in lung, spleen and sperm, miR-1248 in ovary, and miR-1905c in heart, kidney, spleen and testes (Fig. 3).

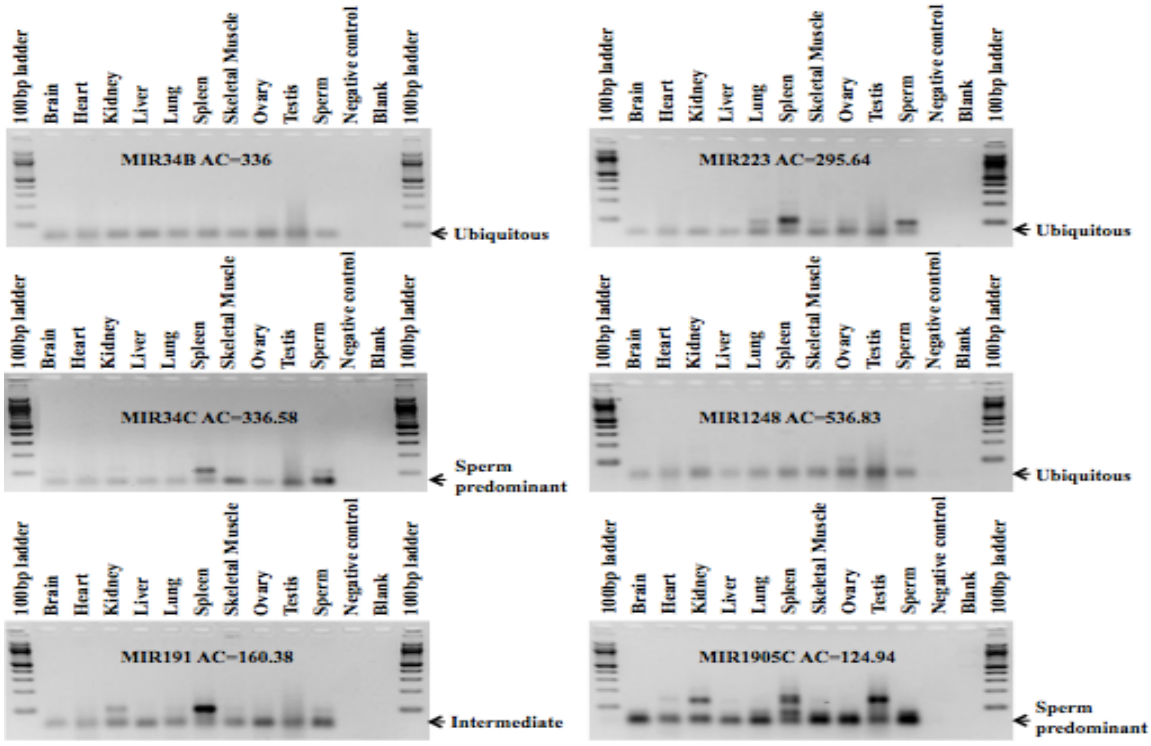

Figure 3. RT-PCR results for miRNA expression in horse tissue panel

Though the RT-PCR analysis was qualitative, the intensity of bands in agarose gel varied indicating varying degrees of expression of these miRNAs in different tissues. For example, though being expressed ubiquitously, the intensity of bands suggested that the number of transcripts of miR-34c and miR-1905c is higher in testes and sperm compared to other tissues. Therefore, the two miRNAs were targeted as potential fertility markers and selected for quantitative PCR experiments in Objective #2.

<u>Objective #2:</u> Quantify expression levels of two sperm-derived and abundant miRNAs, miR-34c and miR-1905c, by qRT-PCR in the sperm and testes of fertile/reproductively mature stallions and subfertile/reproductively immature stallions.

1) Quantitative expression of miR-34c and miR-1905c in the sperm of fertile and subfertile stallions

Through qRT-PCR, we compared the expression levels of sperm/testes-abundant miR-1905c and miR-34c in the sperm of 3 fertile and 3 subfertile stallions. Fertility status of the stallions was previously determined by breeding soundness evaluation and from fertility records (collaborations with Drs. Varner and Love at the department of Large Animal Clinical Sciences). According to this, stallions HS01, HS22, and HS50 were considered fertile and stallions HS03, HS10, and HS21 were subfertile. Our results showed that relative expression level of miR-1905c across all animals was magnitudes higher than the expression of miR-34c, showing fold change values >10,000,000 and >300, respectively (Fig. 4). Comparison of miR-1905c expression between fertile and subfertile stallions did not show clear differences (p = 0.27; Fig. 4 left) between the two groups, though there was a tendency of slightly elevated expression of this

miRNA in subfertile animals. In contrast, expression of miR-34c was significantly higher (p = 0.04; Fig. 4 right) in subfertile stallions compared to stallions with normal fertility.

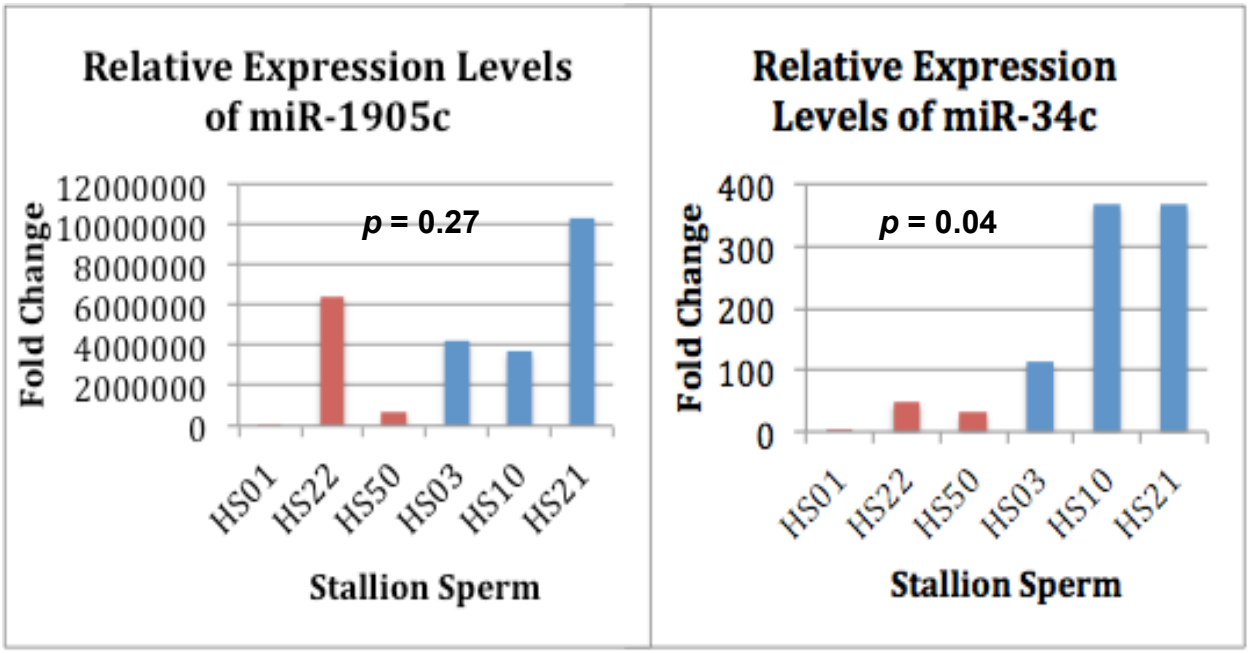

Figure 4. Quantitative (qRT-PCR) expression of miR-1905c and miR-34c in stallion sperm. Fertile stallions (Red): HS01, HS22, HS50; Subfertile stallions (Blue): HS03, HS10, HS21.

2) Qualitative expression of miR-1905c in 1-year old and 3-year old stallions

Because miR-1905c showed high number of transcripts in stallion sperm (Fig. 4 left), expression of this miRNA was further studied in the testes of sexually immature 1-year old and sexually maturing 3-year old stallions. Horses H363, H438, H466, H467, and H468 (Fig. 5 left) were 1-year old stallions and horses H337, H347, H383, H470, and H477 (Fig. 5 right) were 3-year old stallions. The 1-year old stallions were treated as conditional subfertile samples due to the fact

that their testes have not descended yet, and the 3-year old stallions were treated as conditional fertile samples. Fertility of these stallions has not been previously tested and, thus, the fertility phenotypes remain provisional. Qualitative RT-PCR analysis of miR-1905c revealed the presence of this miRNA in both immature and maturing testes with no apparent differences in the amount of PCR products across individual samples (Fig. 5).

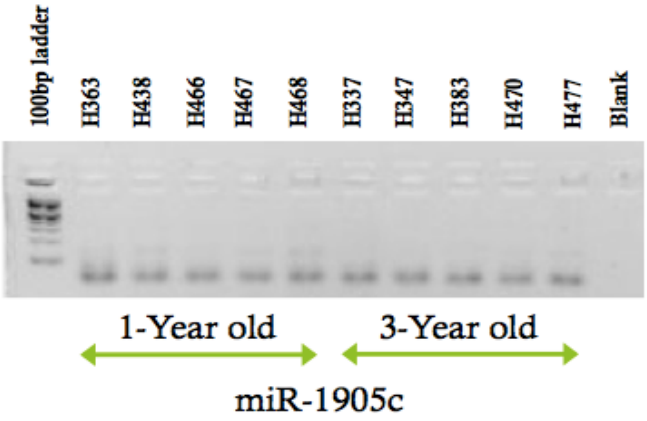

Figure 5. Qualitative analysis of miR-1905c expression in stallion testes. This agarose gel image reveals that miR-1905c is present in stallion testes of sexually immature 1-year old stallions and sexually maturing 3-year old stallions.

3) Quantitative expression of miR-1905c in immature and maturing stallion testes

Through qRT-PCR, expression levels of miR-1905c were quantified in five 1-year old and five 3-year old stallions. No significant differences (p = 0.11) in the expression of miR-1905c were observed between sexually immature and maturing testes, though a tendency of slightly elevated number of transcripts could be seen in samples of maturing testes, particularly in stallion H347 (Fig. 6). The latter most likely reflects individual differences in sexual maturation in stallions.

However, as already mentioned, the fertility phenotypes of the stallions were not verified and cannot conclusively support any hypothesis.

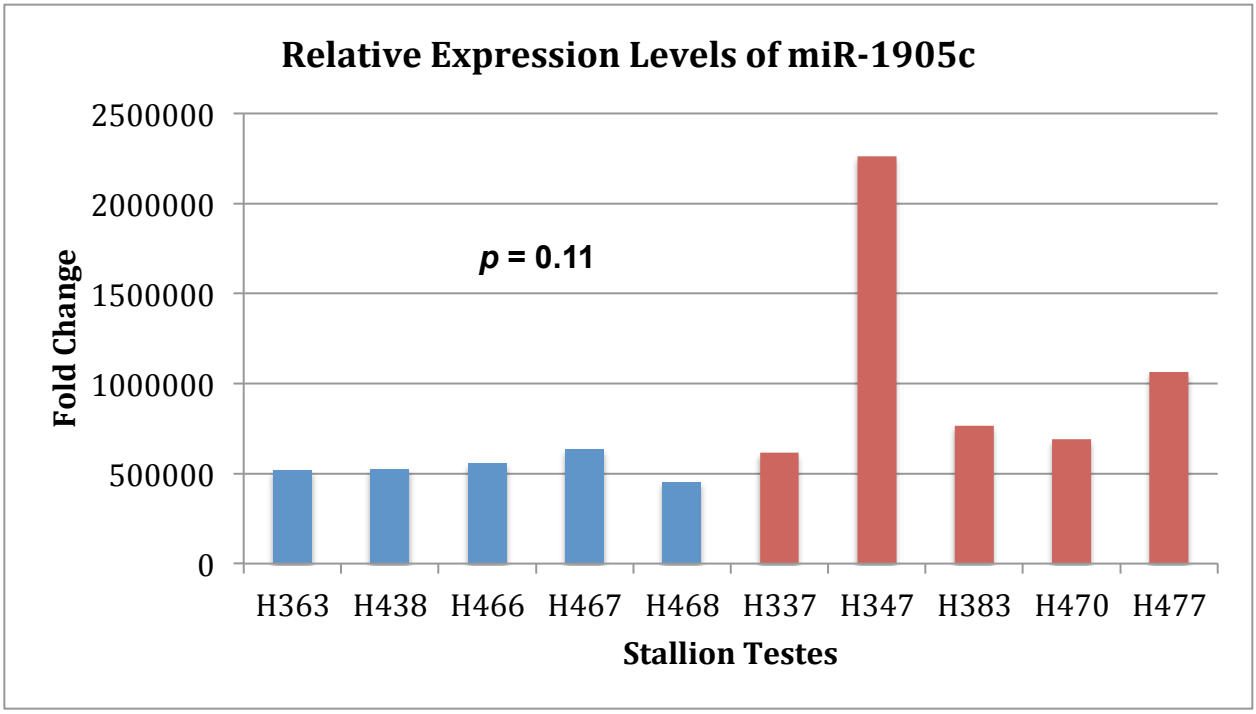

Figure 6. Quantitative expression of miR-1905c and miR-34c in stallion sperm. Immature 1-year old testes (Blue); Maturing 3-year old testes (Red).

<u>Objective #3:</u> Determine the presence of mature-sperm-enriched transfer RNA-derived small RNAs (mse-tsRNAs), a novel class of small regulatory RNAs, in stallion sperm and testes.

Very recently, a novel class of evolutionarily conserved small RNAs was discovered in mouse sperm (Peng et al. 2012). Because these RNAs were highly enriched in mature mouse sperm and were derived from transfer RNAs (tRNAs), they were termed as "mature-sperm-enriched tRNA-derived small RNAs" (mse-tsRNAs). In this objective we examined whether or not mse-tsRNA

Families 1 and 2 are present in stallion sperm and testes. Using the primers specified by Peng and colleagues (2012), and sperm and testes srcDNA samples from one fertile stallion, we showed by qualitative RT-PCR that, indeed, both mse-tsRNA families are present in stallion sperm and testes (Fig. 7). Primers for miR-1905c were used as a positive control. The PCR bands/products obtained with mse-tsRNAs were very similar to those obtained with miR-1905c. Notably, all three primers gave a smaller size product (~ 50 bp) in sperm and a larger size product (~ 70 bp) in testes (Fig. 7). These differences need further investigation by sequencing. In summary, we showed the presence of a novel class of small RNAs in stallion sperm and testes, though the biogenesis and functions of these small RNAs require further research.

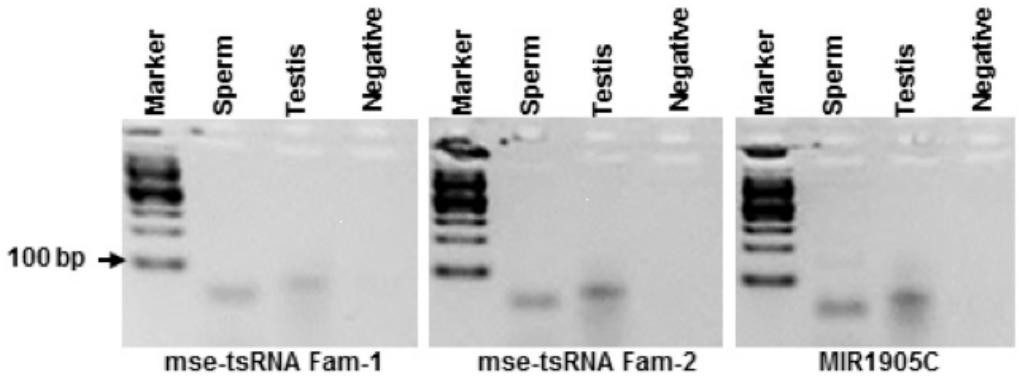

Figure 7. RT-PCR results for mse-tsRNA Family 1 and Family 2 in stallion sperm and testes

CHAPTER IV

CONCLUSIONS

Studies on the involvement of small non-coding RNAs in mammalian fertility and reproduction started only recently (Krawetz et al. 2011) and the first report on the presence of miRNAs in stallion sperm was published just a few months ago (Das et al. 2013). Taken advantage of these findings and considering the proposed regulatory role of small RNAs in sperm functions and early development (Krawetz et al. 2011, Sendler et al. 2013, Das et al. 2013), in this project we examined the expression of six sperm-enriched miRNAs: miR-1905c, -34c, -34b, -1248, -223, and -191 (Das et al. 2013). As a first step, we showed that the expression of these miRNAs was not limited to stallion sperm and their transcripts were also found in a variety of other adult equine tissues, such as brain, heart, kidney, liver, lung, spleen, skeletal muscle, ovary, and testes – an indication of their universal regulatory functions. However, transcription levels of two miRNAs, miR-34c and miR-1905c, were higher in sperm and testes compared to somatic tissues (Fig. 3). Therefore, miR-34c and miR-1905c were chosen as potential biomarkers for stallion fertility and were subjected for further analysis by qualitative and quantitative PCR in the sperm and testes of stallions with variable fertility phenotypes.

Expression of the two miRNAs was examined in the sperm of 3 known fertile stallions and 3 known subfertile stallions, not all of which were of the same breed. While miR-1905c demonstrated equally high levels of expression in all six sperm samples (Fig. 4 left), statistically significant up-regulation of miR-34c was observed in the sperm of subfertile stallions (Fig. 4 right). Though it is tempting to speculate that the higher amount of miR-34c transcripts in the

sperm of subfertile stallions might be related to the role of this miRNA in down-regulating gene expression, the number of samples studied was too limited for drawing any solid conclusions, and further investigation is needed.

Because miR-1905c was so strongly expressed in stallion sperm, its expression was further examined in the testes of five 1-year old and five 3-year old stallions, not all of which were the same breed as well. The fertility in these horses was not verified at the time of the study but the one-year old stallions were treated as conditional subfertile stallions and the three-year old stallions were treated as conditional fertile stallions. This is because in 1-year old stallions, sexual maturation has not begun and the testes have not yet descended, whereas in 3-year old stallions, puberty should have begun and the testes should have descended. Our results showed that though miR-1905c was expressed in the testes of all 10 stallions (Fig. 5), there was no significant difference in the level of miR-1905c expression between sexually immature and sexually maturing testes (Fig. 6). These findings are similar to those observed for miR-1905c expression in the sperm of fertile and subfertile stallions. On one hand, it is possible that the expression level of miR-1905c is not indicative of stallion fertility or sexual maturity. On the other hand, however, it can also be that due to the limited number of samples our statistical analysis could not reveal the actual dynamics of miR-1905c expression in relation to testicular functions. Support to the latter was the elevated expression of this miRNA in a 3-year old stallion H347 (Fig. 6). Whether this is an indication of advanced sexual maturation attributed to individual or breed differences, or just the opposite, a sign of fertility issues, need further investigation. It may be that as stallions sexually mature, slightly increased levels of certain miRNA transcripts are needed to regulate processes related to sexual development. Whereas,

abnormally high quantities of the same miRNAs may change the balance towards down-regulating important genes resulting in reduced fertility.

The mse-tsRNA Families 1 and 2, examined in this study were first discovered in mouse sperm by Peng and colleagues (2012). In this study, they were found to be expressed in stallion sperm and testes as well (Figure 7). This is a novel finding in stallions and shows that these mse-tsRNAs are evolutionarily conserved from a shared common ancestor with mice. This discovery may pave the way for a search for more potential fertility biomarkers that may apply to other mammalian species as well.

Because the number of stallion samples was limited throughout this study, the current results remain inconclusive and further experimentation will be required to solidify any speculations that could be formed from the data of these experiments. However, it is well-established that miRNAs are constantly at work in the mammalian genome, fine-tuning and post-transcriptionally regulating critical processes for the multitude of life functions (Pritchard et al. 2012). The fact that these miRNAs exist not only in somatic tissues in horses but in germ tissues as well supports the idea that in addition to a multitude of crucial functions all over the body, miRNAs are critical regulators also in reproductive cell lines such as sperm (Krawetz et al. 2011). Therefore, the discovery of miRNAs and the knowledge of their functions in the mammalian genome might lead to a new direction in the search of biomarkers for male fertility in a variety of species.

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